Improvement of functional recovery of transected peripheral nerve by means of chitosan grafts filled with vitamin E, pyrroloquinoline quinone and their combination

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ABSTRACT

Effects of vitamin E and pyrroloquinoline quinone on peripheral nerve regeneration were studied using a rat sciatic nerve transection model. Ninety male healthy White Wistar rats were divided into three experimental groups (n = 15), randomly: Sham-operation (SHAM), transected control (TC), chitosan conduit (Chit) and three treatment groups (Vit E, PQQ and PQQ + Vit E). In SHAM group after anesthesia, left sciatic nerve was exposed through a gluteal muscle incision and after homeostasis muscle was sutured. In Chit group left sciatic nerve was exposed the same way and transected proximal to tibio-peroneal bifurcation leaving a 10-mm gap. Proximal and distal stumps were each inserted into a chitosan tube. In treatment groups the tube was implanted the same way and filled with Vit E, PQQ and PQQ + Vit E. Each group was subdivided into three subgroups of six animals each and were studied 4, 8, 12 weeks after surgery. Functional and electrophysiological studies, and gastrocnemius muscle mass measurement confirmed faster and better recovery of regenerated axons in Vit E + PQQ combination compared to Vit E or PQQ solely (P < 0.05). Morphometric indices of regenerated fibers showed number and diameter of the myelinated fibers in PQQ + Vit E was significantly higher than in other treatment groups. In immunohistochemistry, location of reactions to S-100 was clearly more positive than in other treatment groups. Response to PQQ + Vit E treatment demonstrates that it influences and improves functional recovery of peripheral nerve regeneration.

1. Introduction

Peripheral nerve injuries are common in clinical practices,1 and occasionally may result in an acquired life-long disability.2 Grafting is usually suggested for peripheral nerve gaps which are not suitable for neurorrhaphy. The grafts can be autologous, heterologous (allografts) or synthetic biomaterials. Autologous nerve graft is widely accepted, however, it has several disadvantages and donor graft nerves are limited 3; Therefore, various synthetic materials or donor (allografts) or synthetic biomaterials. Autologous nerve graft is usually suggested for peripheral nerve gaps which are not suitable for neurorrhaphy. The grafts can be autologous, heterologous (allografts) or synthetic biomaterials. Autologous nerve graft is widely accepted, however, it has several disadvantages and donor graft nerves are limited3; Therefore, various synthetic materials or synthetic biomaterials.

Recent studies show a benefit of using chitosan as a scaffold in promoting wound healing, cartilage repair and nerve regeneration.4–7 Our previous study showed Chitosan as a suitable functional conduit for promoting of sciatic nerve regeneration in rat.11 Alongside, this temporary guidance tube does not act as a foreign body in situ, also does not affect nerve functional recovery,12 and no needs for second surgery removal.

Recent studies are focused on local application of promoting components such as stromal vascular fraction, stem cells, antioxidants, and biologic agents for peripheral nerve regeneration.13–20 Vitamin E is a primary liposoluble antioxidant. It plays a role in scavenging free oxygen radicals and in stabilizing the cell membranes.21 Degenerative changes in central and peripheral nervous systems have been reported in vitamin E deficiency. Reportedly, nerve regeneration is decreased during peripheral nerve injury in vitamin E-deficient rats.22 Pyrroloquinoline quinone (PQQ) is a low molecular weight redox cofactor that acts as an antioxidant against lipid peroxidation to prevent cell injury. It has been reported to stimulate cell proliferation and to enhance nerve growth factor (NGF) synthesis and secretion in certain cell lines.23,24

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This comprehensive study was performed to evaluate the effects of topically administered Vit E, PQQ and their combination on peripheral nerve regeneration and reinnervation along chitosan conduit repair in rat sciatic nerve transection model. Assessment of the nerve regeneration was based on functional (walking track analysis), electrophysiological measurement, muscle mass measurement, histomorphometric, and immunohistochemical (Schwann cell detection by S-100 expression) criteria at 4, 8, and 12 weeks after surgery.

2. Materials and methods

2.1. Experimental design

Ninety male White Wistar rats weighing approximately 200–240 g, were randomly divided into six experimental groups (n = 15). These groups included sham-operation (Sham group), transected control (TC group), chitosan conduit (Chit group), and three treatment groups. In Chit group, the conduit filled by 20 μl soybean oil (Sigma Aldrich Germany); and in treatment groups the implanted chitosan filled by 20 μl Vit E (20 mg/kg Vitamin E, DL-all-rac-Tocopherol, T3051 Germany) (Chit/Vit E group); 20 μl PQQ (0.03 mmol/lit Pyrroloquinoline quinone, Sigma–Aldrich D7783 China) (Chit/PQQ group), and a combination of 10 μl Vit E + 10 μl PQQ (Chit/Vit E–PQQ combination).

Each group was further subdivided into three subgroups of five animals each. Two weeks before and during the entire experiment, the animals were housed in individual plastic cages (50 × 40 × 20 cm) with an ambient temperature of 23 ± 3°C, stable air humidity, and a natural day/night cycle. The animals were handled on a regular daily basis for 2 weeks prior to the study in order to acclimatize them with testing area and experiment. The rats had free access to standard rodent laboratory food and tap water. All procedures were carried out in accordance with the guidelines of the Ethics Committee, and the University Research Council approved all experiment.

2.2. Preparation of chitosan conduit

A home-made chitosan conduit was constructed based on Qiang Ao et al., 2011. Briefly, chitosan solution was prepared by dissolving medium molecular weight, crab shell chitosan (~400 kDa, 85% deacetylated) (Fluka, Sigma–Aldrich St. Louis, MO, USA) in an aqueous solution (1% w/v) of glacial acetic acid (Merck, Darmstadt, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (at 50 °C) for three hours. The resultant chitosan was filtered through a Whatman No. 3 filter paper. Again, to remove any undissolved particles the solution filtered through vacuum filtration. To overcome the undesired fragile character, glycerol (Sigma Chemical Co., St. Louis, MO, USA) was added as 30% (w/w) of the total solid weight in solution.26 Chitosan conduit was made by gentle injection of the prepared solution into a home-made mold.

2.3. Grafting procedure

Animals were anesthetized by intraperitoneal administration with a combination of ketamine hydrochloride 5%, 90 mg/kg ( Ketaset 5%; Alfåsan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5 mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). Following surgical preparation, in the sham-operation group the left sciatic nerve was exposed through a gluteal muscle incision and after sciatic nerve exposure the splitted muscle was sutured with 4/0 Vicryl (Ethicon, Norderstedt) and the skin with 3/0 nylon (Dafilon, B/Braun, Germany) was closed. In TC group, the left sciatic nerve was exposed the same way and transected proximal to the tibio-peroneal bifurcation where a 8 mm segment was excised, leaving a gap about 10 mm due to retraction of the nerve ends. The proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture and no conduit was placed between the stumps. In Chit group, following nerve transection the proximal and distal nerve ends were inserted 2 mm into the conduit and were sutured with two 10/0 nylon epineurial sutures. In treatment groups, following chitosan conduit implantation the conduit were filled as mentioned before. The ends of conduit were sealed using sterile Vaseline to avoid leakage in all groups. Then, the animals were housed in groups of five per cage under the same condition mentioned above.

The animals of each group were anesthetized by intraperitoneal administration of ketamine–xylazine (see above) and were perfused via left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH = 7.4) at 4°C (n = 5), 8 (n = 5) and 12 weeks (n = 5) after surgery.

2.4. Functional assessment of nerve regeneration

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on Bain et al.27 The lengths of the third toe to its heel (PT), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The Sciatic Function Index (SFI) in each animal was calculated by the following formula:

\[
SFI = -38.3 \times (EPL - NPL)/NPL + 109.5 \times (ETS - NTS)/NTS + 13.3 \times (EIT - NIT)/NIT - 8.8
\]

In general, the SFI oscillates around 0 for normal nerve function, whereas around ~100 SFI represents total dysfunction. The SFI was assessed based on the NC group and the normal level was considered as 0. The SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

2.5. Electrophysiological measurement

After 12 weeks, following the track test, all animals were subjected to electrophysiological studies using Nanoc bio system 320–3760 A trace 80 (USA). Under general anesthesia (see above), the left sciatic nerve was re-exposed by incision of the skin at the previous surgical site. Single electrical pulses (at supra maximal intensity) were delivered via bipolar electrodes placed in turn at the proximal and distal trunk of the regenerated nerve and electromyography (EMG) was recorded by inserting an electrode into the belly of gastrocnemius muscle. The latency and the amplitude of EMG were obtained. Also, the difference in latency of EMG was measured, and the distance between the proximal and distal sites of stimulation was measured to calculate the conduction velocity across the regenerated nerve. On the contralateral, right intact side of each animal, similar measurements were made for the determination of conduction velocity. The conduction velocity of the bridged nerve was expressed as a percentage of that on the intact side of each animal to cancel off variations between animals (%CVR).28

The recovery index of EMG amplitude in all groups calculated based on Suzuki et al. by the formula: Recovery index = Peak amplitude of the operated side/Peak amplitude of the intact side.29

2.6. Muscle mass measurement

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance. All measurements were made by two blinded observers unaware of the analyzed group.
2.7. Histological preparation and morphometric studies

Graft middle cable of Sham, TC, Chit and treatment groups were harvested and fixed with glutaraldehyde 2.5%. The grafts were then embedded in paraplast paraffin, cut in 5 μm and were stained with methylene blue next. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size-related biases.30

2.8. Immunohistochemical analysis observation

Anti-S-100 (1:200, DAKO) was used as marker for myelin sheath. Specimens prior to immunohistochemistry were post fixed with 4% paraformaldehyde for two hours and embedded in paraffin. After non-specific immunoreactions were blocked, sections were incubated in S-100 protein antibody solution for one hour at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for one hour. Horseradish peroxidase-labeled secondary antibody was developed by the diaminobenzidine method. The results of immunohistochemistry were examined under a light microscope.

2.9. Statistical analysis

Experimental results were expressed as means ± SD. All data were analyzed by one-way analysis of variance (ANOVA) to assess statistical significance between experimental groups (SPSS 17.0 for Windows). Dunnett’s test for pair wise comparisons was used to examine the effect of time and treatments. The differences were considered significant when \( P < 0.05 \).

3. Results

3.1. Recovery of sciatic nerve function

The results of SFI values are presented in Table 1. Prior to surgery, SFI values in all groups were near zero. After the nerve transection, the mean SFI decreased to ~100 due to the complete loss of sciatic nerve function in all animals. At the end of the study, the animals of treatment groups achieved a significantly better mean value for SFI compared to that of control group. The statistical analyses revealed that the recovery of nerve function was significantly \((P < 0.05)\) different between chit group and treatment groups. The functional recovery significantly accelerated with application of Vit E, PQQ and their combination in the course of time. Also, this improvement was better with Vit E-PQQ combination compared to Vit E or PQQ solely.

3.2. Electrophysiology

Fig. 1 shows the mean percentage of conduction velocity of the regenerated nerves at the end of experiment. The mean conduction velocity along the regenerated sciatic nerves with application of Vit E, PQQ and Vit E-PQQ combination were 42% CVR, 34% CVR and 54% CVR of the intact right side, respectively. These were significantly higher than chit group which was 17% CVR \((P < 0.001)\). This value were significantly better for groups with application of Vit E \((P < 0.028)\) and Vit E-PQQ combination \((P < 0.016)\) in comparison with PQQ only.

The mean recovery indices for the regenerated sciatic nerves with application of Vit E, PQ and Vit E-PQQ combination were 0.482, 0.312 and 0.5 of the intact right side, respectively. These were significantly higher than chit group which was 0.148 \((P < 0.001)\) (Fig. 2). Also, this value were significantly better for groups with application of Vit E \((P < 0.033)\) and Vit E-PQQ combination \((P < 0.019)\) in comparison with PQQ only. These findings revealed

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**Table 1**

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Sham</th>
<th>TC</th>
<th>Chitosan</th>
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<tbody>
<tr>
<td></td>
<td>Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
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<tr>
<td>Rat 2</td>
<td>–6.1</td>
<td>–2.1</td>
<td>–5.1</td>
</tr>
<tr>
<td>Rat 3</td>
<td>–5.9</td>
<td>–3.9</td>
<td>–4.5</td>
</tr>
<tr>
<td>Rat 4</td>
<td>–6.8</td>
<td>–2.6</td>
<td>–5.5</td>
</tr>
<tr>
<td>Rat 5</td>
<td>–6.1</td>
<td>–3.8</td>
<td>–4.9</td>
</tr>
</tbody>
</table>

(\(\text{Mean ± SD})\)

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**Fig. 1.** Percentage recovery of conduction velocity in treatment groups. Data are presented as mean ± SD. *\(P < 0.005\) vs chitosan group. **\(P < 0.005\) vs PQQ group.
better motor functional recovery of the regenerated nerves in using of Vit E-PQQ combination.

3.3. Muscle mass

Following electrophysiological recordings, all animals were sacrificed for weighting the gastrocnemius muscle and histological and morphometrical analysis. The mean ratios of gastrocnemius muscle weight were measured at the end of the study period (12 weeks after surgery). There was a statistically significant difference of the muscle weight ratios among the treatments and Chit groups ($P < 0.05$). The muscle weight ratios in Vit E, PQQ and Vit E-PQQ combination groups were larger than in Chit group, this value was significantly better for group with application of Vit E-PQQ combination in comparison with Vit E and PQQ; and also is better for Vit E group in comparison with PQQ group. Otherwise, weight loss in gastrocnemius muscle was ameliorated by those topical treatments (Fig. 3).

3.4. Histological and morphometric findings

Table 2 shows the quantitative morphometric analyses of the regenerated nerves for each of the experimental groups. The treatment groups presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness 4, 8 and 12 weeks after surgery, compared to Chit group ($P < 0.05$). On the whole, the histologic and morphometric findings showed Vit E-PQQ combination had more effects on the nerve regeneration than the rest of treatment groups.

**Fig. 2.** Recovery index in treatment groups. Data are presented as mean ± SD. *$P < 0.005$ vs Chitosan group. **$P < 0.005$ vs PQQ group.

**Fig. 3.** Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (injured left and uninjured right) were removed and weighed in the treatment groups at 12 weeks after surgery. Values are given as mean ± SD. *$P < 0.005$ vs Chitosan group. **$P < 0.005$ vs Vit E and PQQ. ***$P < 0.005$ vs PQQ.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of fiber</th>
<th>Diameter of fibers</th>
<th>Diameter of axon</th>
<th>Myelin thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Vit E</td>
<td>1848 ± 142</td>
<td>3847 ± 298</td>
<td>839 ± 101</td>
<td>4.64 ± 0.86</td>
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<td>PQQ</td>
<td>1798 ± 156</td>
<td>3786 ± 287</td>
<td>823 ± 101</td>
<td>4.61 ± 0.86</td>
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<tr>
<td>Vit E-PQQ</td>
<td>1898 ± 15</td>
<td>3904 ± 298</td>
<td>840 ± 101</td>
<td>4.61 ± 0.86</td>
</tr>
<tr>
<td>Sham</td>
<td>1804 ± 146</td>
<td>3824 ± 287</td>
<td>836 ± 101</td>
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<tr>
<td>Chitosan</td>
<td>1796 ± 158</td>
<td>3784 ± 287</td>
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<tr>
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<td>1798 ± 154</td>
<td>3786 ± 287</td>
<td>823 ± 101</td>
<td>4.61 ± 0.86</td>
</tr>
<tr>
<td>Vit E + PQQ</td>
<td>1898 ± 15</td>
<td>3904 ± 298</td>
<td>840 ± 101</td>
<td>4.61 ± 0.86</td>
</tr>
</tbody>
</table>

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**Table 2.** Morphometrical analyses of regenerated nerves for each of the experimental groups: values are given as mean ± SD. *$P < 0.05$ vs Vit E and PQQ. **$P < 0.05$ vs PQQ.
3.5. Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross sections of the regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig. 4). The findings and the expression of S-100 resembled those of the histological evaluations.

4. Discussion

In the present study, the beneficial effects of topically administered Vit E, PQQ and their combination were shown through functional, electrophysiological, morphometrical and immunohistochemical findings in a rat transection sciatic nerve model. Reconstruction of extensive peripheral nerve defects usually needs nerve bridging using conduit or nerve graft techniques. Various organic or synthetic materials have been used as conduit for studying of axonal regeneration in experimental models. Nowadays, conduits are mainly made of non-bioabsorbable materials including silicone or bioabsorbable materials such as aliphatic polyesters, polyurethane, collagen, chitosan and excised artery or vein.2,5,6,31–33

Reportedly, chitosan is a biocompatible and timely biodegradable product which have hemostatic and bacteriostatic properties and low toxicity beside low cost.10,34–37 Chitosan stimulates the tissue repair process and favors angiogenesis.38 It’s degraded products are nontoxic, nonimmunogenic and noncarcinogenic.39 Also, Schwann cells have capacity to adhere and survive on chitosan substratum.40,41 Therefore, these beneficial characteristics convinced us to use this type of conduit for bridging of the experimentally sciatic nerve.

Fig. 4. Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable (A) Sham, (B) TC, (C) VitE/PQQ, (D) CHIT, (E) PQQ, (F) Vit E. There is clearly more positive staining of the myelin sheath-associated protein S-100 (arrows) within the periphery of nerve, indicating well organized structural nerve reconstruction in VitE/PQQ treated nerve compared to that of the SIL group. Scale bar: 10 μm.
gap in present study. Furthermore, the worthwhile capacity of the home-made chitosan conduit was considered in a previous experimental study.23,49

Topical administration of Vit E, PQQ and their combination improved functional recovery of the regenerated sciatic nerve. The results showed, animals of the treatment groups achieved a significant better mean value for SFI compared to Chit group. The highest improvement was identified for Vit E-PQQ combination which may suggest a synergistic effect.

The greater gastrocnemius muscle weight in the treatment groups than Chit group indicated indirect evidence of successful end organ reinnervation in the treated animals. The gastrocnemius muscle weight loss was well ameliorated by topical application of Vit E-PQQ similar to the functional recovery results.

The beneficial effects of topical application of Vit E-PQQ combination and each one were shown by higher mean conduction velocity records along the regenerated sciatic nerves. In this regard, Vit E-PQQ combination was more advantageous than Vit E and the latter was more effective than PQQ. Furthermore, the mean recovery indices for the regenerated sciatic nerves with application of Vit E-PQQ combination, Vit E and PQQ were higher than control group. In this regard, Vit E-PQQ combination and Vit E were more effective than PQQ. In general, these findings suggested the beneficial synergistic effect of motor functional recovery of the regenerated nerves with topical application using of Vit E-PQQ combination.

In general, the quantitative morphometrical indices showed significant improvement of the regenerated nerve fibers in treated animals within the study period. As other assessments, topical application of Vit E-PQQ combination was more effective than other treatments indicating its beneficial synergistic effects on the nerve regeneration.

In immunohistochemistry, the location of reactions to S-100 in treatment groups were clearly more positive than control group further implying that those regenerated axon and Schwann cell-like cells existed and were accompanied by the process of myelination and the structural recovery of regenerated nerve fibers.

The exact mechanism of Vit E and PQQ in enhancement of nerve regeneration is not clear. In the present study, local application of Vit E, PQQ, and Vit E-PQQ combination enhanced sciatic nerve regeneration. Lack of vitamin E in rat diet, potentially contributes to an increase in free radicals. The number of free radicals has also been found to increase as a result of surgical procedures. Reportedly, the axon myelination could deteriorate due to an increase in free radicals and a decrease in antioxidants.22 Otherwise, a reduction of nerve regeneration, size and density of myelinated axons in Vit E-deficit rats is reported.42

Vitamin E as a lipophilic antioxidant protects cell membrane against lipid peroxidation chain reactions,42–45 and strengthens protection against nerve dysfunction caused by oxidative stress.46 Reportedly, Schwann cell growth is greatly stimulated by PQQ. This cell promoter as well as oxidative scavenger could reduce nerve damage by oxidizing the redox modulatory site of N methyl D-aspartate (NMDA) receptors. N methyl D-aspartate receptor has a close relationship with peripheral nerve injuries, which induce NMDA- or glutamate-mediated cell injuries by polysynaptic pathways.47 PQQ can enhance NGF synthesis23,49 from target tissues and Schwann cells,23,49 and this neurotrophic factor demonstrate a significant capacity for improving peripheral nerve regeneration after injury.23,49 It seems local administration of PQQ may also enhance NGF synthesis which contributes to accelerated nerve regeneration.

Maturation and Schwann cells migration into the bridging tube by some neurotrophic factors are essential for the growth of regenerating myelinated axons. As a result of neurotrophic stimulation or contact with a regenerating neurite axolemma, Schwann cells send out cytoplasmic processes that envelope unmyelinated axons. These facilitate axonal nourishment, growth and myelination.47

Significant improvement and acceleration of peripheral nerve regeneration by local administration of Vit E and PQQ into chitosan conduit may result from microenvironment facilities, influence the growth and regenerative capacity of injured neurons.

5. Conclusion

The present study demonstrated that local application of Vit E-PQQ combination accelerate and improve peripheral nerve regeneration after transection of sciatic nerve in rat. These antioxidants are readily available and their topical applications are easily performed without limitations of their probably poor bioavailability in systemic and oral administration.

Ethical approval

The animal care was in accordance with the institution guidelines.

Funding

None.

Author contribution

Saeed Azizi: Study design and writing.
Asghar Azizi: Data collection.
Behnam Heshmatian and Keyvan Amini: Data analysis.

Conflict of interest

None declared.

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